LESSON 24:
BIOCHEMICAL PRODUCTS OF INTEREST – PART I
PHARMACEUTICALS

Objective

Plants are sources of a large variety of biochemicals which are metabolites of both primary and secondary metabolism. But, secondary metabolites are of much greater interest since they have impressive biological activities like, antimicrobial, antibiotic, insecticidal, molluscidal, hormonal properties and valuable pharmacological and pharmaceutical activities, and many are used as flavours, fragrances, colours,etc. Secondary metabolites include a variety of compounds e.g., alkaloids, terpenoids, phenyl propanoids etc. This Lesson gives a detailed account of the various secondary metabolites obtained through plant tissue culture which are of pharmaceutical interest.

Pharmaceuticals

Plant secondary metabolites can be divided into several groups as follows:

Alkaloids

A variety of alkaloids have been used as pharmaceuticals and most of them are plant metabolites. The typical tropane alkaloids, atropine, hyoscyamine, scopolamine and cocaine, were widely used as blockers of the parasympathetic nervous system such as anodyne and antispasmodic. Research on production of these useful alkaloids by plant cell cultures has been carried out for more than 25 years, however, industrial production has not yet succeeded because of low producing ability of the cultured cells. Plants used for these studies are mainly Atropa belladonna, Hyoscyamus niger, Datura meteloids and others.

Various approaches to increase their productivity have been tried by many researchers as briefly described in the previous section. At present, vinblastine, an antitumor alkaloid, is most likely to be produced commercially by a Japanese company using a combination process of plant cell culture and that of chemical synthesis which was initially investigated by a Canadian company, Allelix Inc.

Morphinan Alkaloids

Codeine is an analgesic and cough-suppressing drug and Papaver somniferum L. (opium poppy) is a traditional commercial source of codeine, and morphine which can be converted to codeine. Mature capsules of P. bracteatum accumulates up to 3.5% of thebaine which also can be converted to codeine. Although many researchers have tried to produce codeine by undifferentiated cells of these plants, little success has been achieved.

Kamimura et al. indicated that morphogenetic differentiation from cultured cells of P. bracteatum was prerequisite for producing higher levels of thebaine. Staba et al., Constabel et al., Furuya et al., Kamo et al. and other researchers also showed the similar results as Kamimura's report using P. bracteatum and P. somniferum. For example, Furuya et al. reported that cells of P. somniferum produced norsanguinarine, sanguinarine, cryptopine and other various alkaloids, but not codeine and morphine.

Concerning sanguinarine, Eilert et al. showed that fungal mycelium of Botrytis sp. elicited production of sanguinarine by P. somniferum cells. The level of this alkaloid increased 26 times in the presence of the elicitor, 29% of the dry cell weight, compared to the medium without it. Since the alkaloid extracted from intact plants is added to toothpastes as an anti-plaque agent, the commercial production of sanguinarine by cell culture technology was intensively investigated by Kurz's group at the National Research Council of Canada and a company in the U.S. several years ago, but the process has not yet been commercialized.

The efficient production of thebaine and codeine using cell culture systems by de novo synthesis was not successful, Furuya et al., therefore studied the biotransformation of codeinone to codeine using immobilized cells of P. somniferum. The conversion yield was 70.4% and about 88% of codeine converted was excreted into the medium.

Berberine

Berberine is an isoquinoline alkaloid which is distributed in roots of Coptis japonica and cortex of Phellondendron amurense. Berberine chloride is used for intestinal disorders in the Orient and it takes 5 to 6 years to produce Coptis roots as the raw material.

Furuya at Kitasato University and Yamamoto at Nippon Paint have investigated the production of berberine by Coptis japonica cell cultures since 1970's and Yamada et al. at Kyoto University selected a high berberine producing cell line of C. japonica which was transferred to a Japanese company, Mitsui Petrochemical.

Mitsui Petrochemical has improved the productivity, and Hara et al. found that addition of 10^{-8} M gibberellic acid into the medium stimulated berberine productivity up to 1.66 g per L of the medium. Using a cell sorter and protoplasts of C. japonica, they selected many higher alkaloid-producing cell lines. Thus, the Mitsui group produces berberine in a large scale at a level of 1.4 g per L of their optimized medium within 2 weeks. Furthermore, they established a "high-density cell culture" process to produce berberine much more efficiently. In order to achieve a cell mass of 70 g/L on a dry weight basis, stirring without damaging the cells, supply of sufficient amounts of oxygen, and that of appropriate nutrients were optimized. As a result, the yields reached 70 g per L of cell mass and 0.45 g/day of berberine. The continuous culture with high cell density was also conducted successfully.

Addition of a polyamine, spermidine, was found to stimulate the production of berberine by Thalictrum minus cell suspension cultures by Hara et al. in Tabata's laboratory although other polyamines such as cadaverine, putrescine and spermine

were ineffective. They indicated that spermidine effected an increase of ethylene generation which was associated with activation of berberine synthesis. The maximum stimulative effect was obtained by addition of 2 mM spermidine.

Tropane Alkaloids

Scopolamine and hyoscyamine are being used commercially as anesthetic and antispasmodic drugs. These alkaloids occur in leaves of (Solanaceae) plants including D. myoporoides and D. leichhardtii. Scopolia, Atropa, Hyoscyamus and Datura also contain tropane alkaloids.

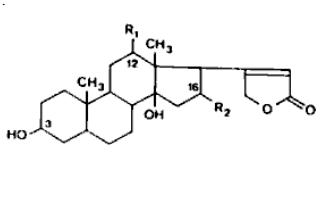
Studies on production of tropane alkaloids by plant tissue cultures have been actively carried out by many researchers since West et al. found tropane alkaloids in an Atropa belladonna callus more than 30 years ago. However, the concentrations of scopolamine and hyoscyamine in cultured cells are generally very low in spite of many efforts to increase the yield using various approaches. Therefore, the plant cell culture has not yet been employed to manufacture these alkaloids. For example, Tabata et al. added tropic acid into Scopolia japonica suspension cultures as a precursor and could increase the level of alkaloids up to 15 times. Mitsuno et al. selected a high tropane alkaloid producing strain of Hyoscyamus niger which produced about 7 times more hyoscyamine ($13.9X10^{-3}$% fresh cells), than that of the parent strain. According to their results, there was no direct correlation between high producing ability and variation of chromosomal numbers.

Endo et al., Kitamura et al. and many other scientists reported that roots differentiated from cultured cells accumulate scopolamine, hyoscyamine and/or nicotine. However, Kitamura indicated that the alkaloids were not accumulated in leaves of the regenerated plantlets.

Since the alkaloids are synthesized in the root, Flores et al. cultivated hairy roots transformed with Agrobacterium rhizogenes and showed the production of hyoscyamine and other alkaloids at the similar levels to the normal roots.

Cardinolides

Cardiac glycosides or cardenolides are products of Digitalis species. Some of these compounds have been employed in treatment of heart diseases. Chemical structures of the major aglycone of cardenolides are shown in Fig.1. and various sugar residues link the 3-hydroxy group of aglycones (Table 1). For commercial production Digitalis plants are being cultivated in the fields of several countries including the Netherlands, Hungary and Argentina. A digoxin product, Lanoxin, is a brand name of a Burroughs Wellcome product and has the largest market of the company's cardiovascular drugs. The major markets of Lanoxin are in the U.S.A. and Italy, and the total sales are approximately 6000 kg a year with 50 million U.S. dollars. Other companies such as Boehringer Mannheim, Merck Darmstadt and Beiersdorf AG in Germany also sell cardiac glycosides.



	R_1	R_2
DIGITOXIGENIN	H	H
GITOXIGENIN	H	OH
GITALOXIGENIN	H	OCHO
DIGOXIGENIN	OH	H
DIGINATIGENIN	OH	OH

Fig. 1. Principal Cardioactive Glycosides of Digitalis species: Chemical Structures

The studies of plant tissue and cell cultures for production of cardiac glycosides were begun more than 30 years ago and the report presented by Hildebrandt and Riker in 1959 was probably the first one in this field. Staba investigated the nutritional requirements of tissue cultures of Digitalis lanata and D. purpurea in 1962. These two species are being commonly used by many scientists.

Although there are a number of papers describing production of cardiac glycosides in Digitalis tissue cultures, generally the yield was very low, and moreover, during the successive transfers of the cultured cells the amount of cardenolides often decreased and disappeared completely.

Instead, many researchers indicated that morphological differentiation caused an increase in productivity. For example, Lui and Staba showed that organ cultures of D. lanata leaves and roots produced cardenolides and during the cultivation, the level of digoxin in the tissues rose with increasing age. Hirotani and Furuya also found that renewed organ differentiation from callus tissues of D. purpurea led to a new formation of cardinolides.

Nutritional factors including growth regulators, sugars, nitrogen sources, vitamins and so on in the medium affect on differentiation of shoots and other organs and the secondary metabolites such as cardiac glycosides are often synthesized. Hagimori et al. of Japan Tobacco Inc. cultivated shoot-forming tissues of D. purpurea in a 3 L jar fermentor and detected high concentrations of cardiac glycosides including digitoxin. The differentiated tissue culture is prerequisite for secondary metabolites production in some cases, however in general, the method requires much longer culture time than the suspension cell culture and consequently it is not efficient.

Various secondary metabolites other than digitoxin and digoxin have been found in callus tissues of D. lanata and D. purpurea. These include cholesterol, campesterol, stigmasterol, ß-sitosterol, 4-hydroxy-digitolutein and others. However, as described above, Kartning found that the levels of those secondary products tended to decrease over several passages in cultivation.

In contrast to the de novo synthesis, the biotransformation process with Digitalis plant cells seems to be more promising from a commercial point of view. Graves and Smith reported that D. lanata and D. purpurea callus cultures rapidly transformed progesterone to pregnane. Leaf and root cultures of D. lanata and shoot-forming callus tissues of D. purpurea accumulated an increased level of digoxin and/or digitoxin when progesterone was added to the cultures. Stohs and Staba

studied on the biotransformation of cardenolides by Digitalis cells and recognized that the glycosylation reaction occurred.

Among many studies, the biotransformation from digitoxin to digoxin using Digitalis lanata cells investigated by Reinhard and Alfermann is the most interesting approach in terms of commercial application since digoxin has a higher demand as a drug for heart diseases than digitoxin. It is advantageous that Digitalis leaves contain a larger amount of digitoxin which can be used as a substrate. It is a hydroxylation reaction at the 12 ß-position of digitoxin, and Reinhard et al. found that ß-methyldigitoxin was the most suitable substrate in this biotransformation as methyldigoxin is the major product. To reduce the production cost, the same group examined the use of immobilized D. lanata cells and semi-continuous culture. The result of a typical biotransformation for 17 days using a 20 liter reactor is shown in Table 2. As described previously, the process was tested in large scale reactors for commercial application by Boehringer Mannheim Co. in Germany. However, the process, so far, has not yet been industrialized.

Table 1. Principal Cardioactive Glycosides of Digitalis species
Overview of Sugar Residuals Glucosidically Linked to the 3-Hydroxy Group

PLANTS	GLYCOSIDES	SUGARS
Digitoxigenin	Lanatoside A Glucodigifucoside Glucoevatromonoside Purpurea glycoside A Digitoxin	Gl-Acdx-Dx-Dx- Gl-Fuc- Gl-Dx- Gl-Dx-Dx-Dx- Dx-Dx-Dx-
Gitoxigenin	Lanatoside B Digitalinum verum Glucogitoroside Purpurea glycoside B Gitoxin	Gl-Acdx-Dx-Dx- Gl-Dtl- Gl-Dx- Gl-Dx-Dx-Dx- Dx-Dx-Dx-
Digoxigenin	Lanatoside C Digoxin	Gl-Acdex-Dx- Dx- Dx-Dx-Dx-
Diginatigenin	Lanatoside D Diginatin	Gl-Acdx-Dx-Dx- Dx-Dx-Dx-
Gitaloxigenin	Lanatoside E Glucoverodoxin Glucolanadoxin Glucogitaloxin Gitaloxin	Gl-Acdx-Dx-Dx- Gl-Dtl- Gl-Dx- Gl-Dx-Dx-Dx- Dx-Dx-Dx-

Acdx = 3-acetyl-ß-D-digitoxose; Dtl = ß-D-Digitalose; Dx = ß-D-Digitoxose; Fuc = ß-D-Fucose; Gl = ß-D-Glucose

Table 2. Biotransformation of ß-Methyldigitoxin to ß-Methyldigoxin by Digitalis lanata Cells in a 20 L Reactor

ß-Methyldigitoxin added	17.24 g	(100 %)
Unconverted ß-methyldigitoxin	2.04 g 14.36 g	(11.8%) (81.7%)
ß-Methyldigoxin formed	0.28 g	(1.4%)
By-product		
Yield	94.90%	

L-DOPA

L-DOPA, L-3,4-dihydroxyphenylalanine, is an important intermediate of secondary metabolism in higher plants and is known as an precursor of alkaloids, betalain, melanine, and others. It is also a precursor of cathecolamines in animals and is being used as a potent drug for Parkinson's disease.

Brain in 1976 found that the callus tissue of Mucana pruriens accumulated 25 mg/L DOPA in the medium containing very high concentration of 2,4-D. Wichers et al. immobilized cells of M. pruriens within alginate and found that the cells produced DOPA from tyrosine in up to 2% of dry cell weight. The DOPA synthesized was secreted mostly into the medium.

Teramoto and Komamine induced callus tissues of Stizolobium hassjoo (Mucuna hassjoo), M. pruriens and M. deeringiana, and optimized the culture conditions. The highest concentration of DOPA was obtained when S. hassjoo cells were cultivated in MS medium containing 0.025 mg/l 2,4-D and 10 mg/l kinetin. The level of DOPA in the cells was about 80 nmol/g-f.w.

Valepotriates

Plants in the Valerianaceae have been used as folk medicines. For example, Nardostachys jatamansi, Valeriana wallichii and V. officinalis L. var. angustifolia have been used in India, and N. chinensis has been employed in China for hundreds of years. Partrinia plants are also being used as sedative drugs in former Soviet Union. Although the active principles in these plants have not been identified, a group of compounds having biological activities such as sedative, tranquilization, cytotoxicity and antitumor activities were named "valepotriates". Thies synthesized a series of valepotriate derivatives and tested for biological activities.

Becker et al. have investigated plant tissue cultures for producing valepotriates because of their limited supply and uncertain availability. They induced callus tissues of nine different species of Valerianaceae on MS media and found that Fedia cornucopiae and V. locusta cells produced higher levels of the compounds than the intact plants. Isolation of cell lines resistant to trifluoroleucine and to nystatin, treatment with colchicine, cultivation of the cells in two-phase culture media and addition of several bioregulators were carried out intensively. As valepotriates have monoterpene skeletons, L-leucine was considered as a precursor. Baker et al. isolated cell lines of V. wallichii resistant to trifluoroleucine, a leucine analog, but the yield of valepotriates in the cells was not increased although the intracellular level of leucine was increased. It was reported that fungi resistant to nystatin, a polyene antibiotic, produced a high

level of steroids. One of the strains of V. wallichii resistant to nystatin increased the amount of valepotriates produced up to 3 times, which was 88 mg/g-d.w. The same group treated a suspension culture of V. wallichii with colchicine which was expected to induce polyploid cells. As a result, they found that the colchicine treated cells produced higher amount valepotriates than the respective untreated cultures. A two-phase culture provided by addition of RP-8 (Merck) into the culture medium was effective in inducing secretion of the lipophilic compounds from the cells, and the total yield of valepotriates was substantially increased.

Since the valepotriate skeleton is of the iridoid nature, they added some plant bioregulators such as dimethyl-morpholinium-bromide, dimethyl-piperidinium-bromide, dimethyl-piperidinium-chloride as well as 2-(3,4-dichloro-phenoxy)-triethylamine and 2-(3,5-diisopropylphenoxy)-triethylamine to cell suspension cultures of F. cornucopiae and V. wallichii. When they were employed in concentrations of 0.01 to 0.04 mmol during the early exponential growth stages of the cells, the levels of valepotriates were increased significantly.

Antitumor Compounds

The plant kingdom is one of the attractive sources of novel antitumor compounds. The National Cancer Institute in the U.S., for example, has conducted an intensive screening program since 1955 and has identified various potent compounds from higher plants. These antitumor compounds include maytansine, tripdiolide, homoharringtonine, bruceantin, ellipticine, thalicarpine, indicine-N-oxide, and baccharin. In addition to these compounds, some of the higher plant products such as vinblastine, vincristine, podophyllotoxin derivatives including etoposide, and camptothecin and its derivative have already been marketed as very important anticancer drugs. Taxol from Taxus brevifolia and related plants, is one of the most exiting compounds and was marketed in 1992.

However, the concentrations of these active compounds in plants are generally low (Table 3.), the growth rate of the plants is slow and the accumulation pattern of these compounds is highly susceptible to geographical or environmental conditions. Therefore, it is not an easy task to produce economically these compounds by extraction from intact plants. Furthermore, as indicated for taxol production, the exhaustion of native producing-plants is becoming a serious problem in terms of environmental preservation.

Table 3. Antitumor Compounds Isolated from Higher Plants

Antitumor Compounds	Plant (dry wt. %)
Baccharin	2.0×10^{-2}
Bruceantin	1.0×10^{-2}
Camptothecin	5.0×10^{-3}
Ellipticine	3.2×10^{-5}
Homoharringtonine	1.8×10^{-5}
Maytansine	2.0×10^{-5}
Podophyllotoxin	6.4×10^{-1}
Taxol	5.0×10^{-1}
Tripdiolide	1.0×10^{-3}
Vinblastine, vincristine	5.0×10^{-3}

Though plant tissue culture processes are still not cost-effective if targeted products could easily be manufactured by chemical, fermentative and/or extraction processes, this technology is undoubtedly one of the appropriate approaches to solving the above-described problems. And many interesting antitumor compounds isolated from higher plants have very complicated chemical structures. Therefore, the research interest has grown for over a decade and a couple of processes based on the plant tissue culture technology are likely to be applied for commercial production of antitumor drugs.

The following are examples of the technology.

Camptothecin

Camptotheca acuminata, a native of North China, was found to produce a potent antitumor alkaloid, camptothecin, by Wall et al. in 1966. It is highly active in Walker 256 rat carcinosarcoma and mouse leukemia, p388 and L1210. The clinical trials in patients with gastrointestinal cancer were at first very promising but subsequent trials showed toxicity.

Sakato and Misawa induced callus from the stem of C. acuminata on MS solid medium containing 0.2 mg/L 2,4-D and 1 mg/L kinetin. The callus was transferred to the liquid medium. Gibberelin, L-tryptophan and conditioned medium stimulated growth of the cells. After 15 days of cultivation in suspension, the concentration of camptothecin in the cells was 0.0025% on a dry weight basis, which was about 1/20 of the level in the intact plant. A.J. van Hengel et al. in the Netherlands established the suspension culture system of C. acuminata and detected camptothecin in the cultured cells using TLC, HPLC and GC-MS. The highest level, 0.998 mg of camptothecin per liter of the medium, was accumulated in the cells cultivated in MS medium containing 4 mg/L NAA.

10-Hydroxycamptothecin having less toxicity is so far a promising derivative of camptothecin and is in clinical trials in the U.S.

Homoharringtonine

Homoharringtonine together with harringtonine and isoharringtonine, were isolated from Cephalotaxus harringtonia by Powell et al. in 1969. These alkaloids are complex esters of the inactive alcohol, cephalotaxine. These compounds inhibit the growth of murine leukemias, L1210 and P388, and KB cells. Homoharringtonine also shows activity against colon tumors, melanoma and leukemia in mice.

Since the chemical synthesis of homoharringtonine was not efficient for commercialization, studies of tissue culture were conducted by Delfel and his colleagues. They detected about 5-10 mg of the alkaloids per kg dry wt. of the callus tissues cultivated for 3 to 6 months. The levels were approximately 1 to 3% of the concentrations found in the parent plant. These products were cephalotaxin, homoharringtonine, harringtonine and isoharringtonine.

MS medium containing 1 mg/L kinetin and 3 mg/L NAA was favorable to induce the callus from C. harringtonia, while the medium without growth regulators strongly promoted organogenesis. The radioimmunoassay established by the same group showed the levels of cephalotaxine and its esters were only 1/300 of the intact plant.

Podophyllotoxin

Podophyllum pelatatum, May apple, which is a common herb in eastern North America contains an antitumor lignan, podophyllotoxin. It is active to KB cells and is used against certain virus diseases and skin cancer. A semi-synthetic derivative of podophyllotoxin, etoposide (V-16), was found to be active against brain tumor, lymphosarcoma and Hodgkins' disease and was approved by the FDA in the U.S. Bristol-Myers Squibb is one of the largest manufacturers of the drug.

Production of podophyllotoxin by P. pelatum cell cultures was first attempted by Kadkade and he found that a combination of 2,4-D and kinetin in the medium supported the highest amount of its production. Red light stimulated the production.

Sakata et al. of Nippon Oil induced embryogenic roots from a callus of the plant in a liquid MS medium supplemented with 1 mg/L NAA, 0.2 mg/L kinetin and 500 mg/L casein hydrolysates. The roots were then transferred to the medium without growth regulators. They detected 1.6% of podophyllotoxin in the dried tissues, which was 6 times higher level than that in a mother plant.

To increase the yield of podophyllotoxin, Woerdenberg et al. in the Netherlands added a complex of a precursor, coniferyl alcohol, and ß-cyclodextrin to Podophyllum hexandrum cell suspension cultures. Addition of 3 mM coniferyl alcohol complex gave 0.013% podophyllotoxin of the cells on a dry weight basis but the cultures without the precursor produced only 0.003%. ß-D-glucoside of coniferyl alcohol, coniferin, was a more potent precursor in terms of the yield of the anticancer compound (0.055%), but unfortunately this compound is not commercially available. The same authors reported that cell suspension cultures of Callitris drummondii (conifer) also accumulated podophyllotoxin-ß-D-glucose. In the dark, the cells produced approximately 0.02% podophyllotoxin of the dry cell mass and 85-90% of the lignans were the ß-D-glucoside form, while in the light the yield of podophyllotoxin-ß-D-glucose increased to 0.11%.

Smooly et al. reported that callus tissues and suspension culture cells of Lilium album produced podophyllotoxin. One of the cell lines produced 0.3% podophyllotoxin of dried cells together with small amounts of 5-methylpodophyllotoxin, lariciresinol and pinoresinol after 3 weeks of cultivation. The callus tissue induced from P. hexandrum was reported by Heyenga to produce podophyllotoxin, 4'-demethyl-podophyllotoxin and podophyllotoxin-4-0-glucoside when the callus was incubated in B5 medium containing 2,4-D, gibberellic acid and 6-benzylaminopurine. The levels of podophyllotoxin and its derivatives were similar to those in the mother plant.

Vinca Alkaloids

The dimeric indole alkaloids, vinblastine and vincristine have become highly valued drugs in cancer chemotherapy due to their potent antitumor activity against various leukemias, Hodgkin's disease and solid tumors. They are currently produced commercially by extraction from Catharanthus roseus (Apocyanaceae) plants, but the process is not efficient because of very low concentrations of the alkaloids in the plant. It was reported that the concentration of both vinblastine and vincristine was only 0.0005% as a dry weight basis.

In order to produce these useful anticancer drugs much more efficiently, many scientists have tried to apply plant tissue culture technology. In fact, a large number of papers related to this approach have been presented since the first research carried out by Carew et al. in 1966. However, production of both alkaloids by de novo synthesis using the callus or the suspension cultured cell of C. roseus is so far not promising because the productivity of the cultured cells reported was so far very low.

Misawa and his colleagues of Allelix Inc. in Canada studied on production of vinblastine by an alternative way in collaboration with Kurz of the National Research Council of Canada and Kutney of University of British Columbia, and established an economically feasible process consisting of production of catharanthine by plant cell fermentation and a simple chemical or an enzymatic coupling.

The vinblastine molecule is derived from two monomeric alkaloids, catharanthine and vindoline as shown in Fig.2. The concentration of vindoline in the intact C. roseus plant is approximately 0.2% as a dry weight basis, which is much a higher level than catharanthine, and the cost of vindoline is less expensive compared to catharanthine and vinblastine. The Allelix group, therefore, investigated the production of catharanthine by a cell suspension culture process with a selected C. roseus cell line induced from anthers on Gamborg's B5 medium containing 2% sucrose, 1 mg/L 2,4-D and 0.1 mg/L kinetin. The cells were grown in 250 ml flasks containing 60 ml of MS liquid medium supplemented with 3% sucrose, 1 mg/L NAA and 0.1 mg/L kinetin under continuous diffuse light on a rotary shaker (250 r.p.m.) at 25oC. In experiments for optimization of catharanthine production, they transferred 7 day old cells to a test medium and subcultured for 3 passages. In the 4th passage, 60 ml cultures were harvested in triplicate after 2 or 3 weeks growth, and the cell mass and alkaloid content were determined.

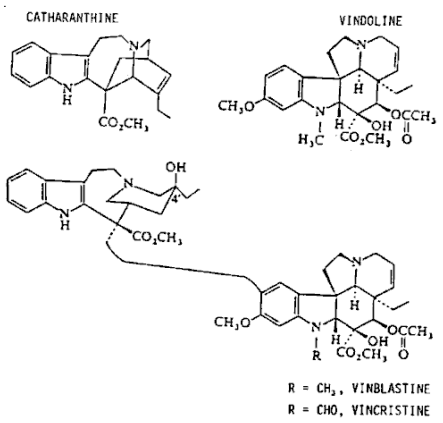


Fig. 2. Chemical Structures of Catharanthine, Vindoline, Vinblastine and Vincristine

The results showed that the MS medium was the most favourable for catharanthine production but the optimal levels of phytohormones for the growth and the production were varied in different cell lines. For example, one line required no phytohormones but another line required 0.1 mg/L NAA and 0.1 mg/L kinetin. Addition of various chemically defined compounds to the medium as "inducers" was found to stimulate the production efficiently. Among them effects of vanadyl sulphate, abscisic acid and NaCl on the production of catharanthine were significant. Based on the conditions optimized by using flasks, Smart et al. scaled up the cultures to 10, 30 and 100 L-air lift fermentors. When abscisic acid was added to the culture as an elicitor on the 7th day of cultivation, the final titer of catharanthine was raised to 85 mg/L in a 30 L fermentor.

The second stage in this project, the Allelix's group tried to couple enzymatically or chemically catharanthine produced by the cell culture process with commercially available vindoline. As an enzyme source for the coupling, a crude preparation obtained by 70% ammonium sulphate precipitation from the cultured cells of C. roseus was used. The reaction mixture containing both monomeric alkaloids, Tris buffer (pH 7.0) and the enzyme preparation was incubated at 30oC and for 3 hours. It was determined that the enzyme reaction gave various dimeric alkaloids including vinamidine, 3-(R)-hydroxyvinamidine and 3'4'-anhydrovinblastine. Leurosine and catharine, oxidized derivatives of anhydrovinblastine, were also detected in the early stages of the incubation. They found that $MnCl_2$ and either FAD or FMN stimulated the coupling. Although neither vinblastine nor vincristine was detected in the mixture, it was recognized that a substantial amount of anhydrovinblastine was formed as a major coupling product when an excess amount of sodium borohydride was added to the mixture after incubation.

In order to investigate properties of the coupling enzyme(s) it was partially purified with gel filtration and isoelectric focusing and five isozymes were obtained by Endo et al. One of them had MW 15,000 and the other four had the same MW (37,000). All of these isozymes were shown to have peroxidase activity. Using the partially purified enzymes, anhydrovinblastine was formed with a conversion yield of about 50%.

Formation of vinblastine from vincristine as detected by Goodbody et al. using a crude enzyme preparation obtained from suspension cultured cells of C. roseus. The highest yield of conversion obtained was 13% from 0.13 mg anhydrovinblastine in 1 ml of the reaction mixture after 3 hours incubation at 30oC, pH 7.0.

During the course of these studies on coupling mechanisms, they found that ferric ion catalyzed the coupling reaction significantly in the absence of the enzyme. It is of interest that the products of the chemical coupling were not only anhydrovinblastine but also vinblastine. The yields of both alkaloids were 52.8% and 12.3%, respectively after 3 hours incubation at 30oC, pH 7.0. These products including catharanthine were analyzed by high resolution mass spectrometry as further confirmation of their identification. Circular dichroism confirmed that a-coupling exists between the 2 monomeric units of both vinblastine and vincristine produced either enzymatically or chemically.

This is a novel and an efficient process to produce an antitumor drug, vinblastine, and is likely to be applied commercially. The technology was transferred from the Canadian company to a Japanese company, Mitsui Petrochemicals Industry for further development.

Hara et al. of Mitsui Petrochemical could increase the yield of catharanthine up to 150 mg/L in the MS medium supplemented with 1 mg/L NAA and 0.1 mg/L kinetin using the best producing cell line isolated from Allelix's cell line. The stimulating activity of NaCl and KCl on alkaloid production was also confirmed. Furthermore, the scientists of Mitsui employed high-cell density cultures and reported yields of catharanthine of 230 mg/L/week.

The yield of vinblastine by the chemical coupling reaction was also improved by the same group; addition of ferric chloride, oxalate, maleate and sodium borohydrate stimulated the yield of vinblastine from anhydrovinblastine up to 50%.

Bede et al. also investigated the production of anhydrovinblastine. They employed a two-enzyme system containing horseradish peroxidase and glucose oxidase to catalyze the formation of anhydrovinblastine from catharanthine and vindoline. Although peroxidase requires hydrogen peroxide for the coupling reaction, its presence in excess in the reaction mixture may inhibit the reaction. But addition of glucose oxidase was used to allow the controlled, continuous production of hydrogen peroxide at low levels, minimizing oxidative reactions. Both enzymes were immobilized on Euperight C beads, an oxirane matrix, and the system was shown in catalyze the coupling reaction.

Taxol

Under the intensive NCl screening program of antitumor compounds in the U.S., Wall et al. began to isolate an active principle against KB cells from a tree, Taxus brevifolia in 1965. In 1969 pure taxol was first isolated and its chemical structure was disclosed in 1971. It is a diterpene amide and has shown

against B16 mouse melanoma tumor, the MX-1 human mammary xenograft and CX-1 colon xenografts. The mode of action of taxol is rather unique because it stabilizes microtubles and inhibits depolymerization. The clinical trials begun in 1983 have shown positive results in the treatment of advanced ovarian cancer and breast cancer as well.

The FDA in the U.S. has approved taxol (generically known as paclitaxel) at the end of 1992; Bristol-Myers Squibb produces the drug for use in the treatment of ovarian cancer in patients who have failed to respond to other chemotherapies. Taxol is now being manufactured by extraction from the bark of wild-grown T. brevifolia trees. The demand for taxol will be undoubtedly increasing since it will be applied for other cancers including breast and lung cancers in the near future, but its supply is limited. Other related plants such as T. canadensis and T. cuspidata also contain taxol and other related compounds. Generally the concentration of taxol in Taxus plants are very low. Therefore, harvesting Taxus trees for production of taxol commercially causes a serious problem in the U.S. from the environmental point of view.

As alternative ways, plant tissue and cell cultures as well as chemical transformation processes from baccatin extracted from needles of the plant and the total chemical syntheses have been investigated in many research groups. In the U.S., Phyton Catalytic Inc. and ESCAgenetics announced a couple of years ago that they were establishing plant cell culture processes to manufacture taxol. The latter showed a photograph of a vial containing taxol powder produced by plant cell cultures. However, both companies have never published details of the plant cell culture procedure in scientific periodicals or at any meetings.

A couple of patents have been published. For example, a U.S. Patent filed by A.C. Christen et al. of U.S.D.A. described that the tissue of T. brevifolia had been successfully cultured to produce taxol, related alkaloids and alkaloid precursors, but the patent claims are exceedingly broad and identity of what the actual process was remains difficult to ascertain.

Shuler et al. at Cornell University showed that a cell line of T. brevifolia in suspension provided by USDA-Agriculture Research Station and Phyton Catalytic, produced 3.9 mg/L taxol in the medium after 26 days of culture. The level of taxol was determined by reverse-phase HPLC. They found that fresh cell weight increased by 4-fold after a lag phase of about 4 days and that taxol was first seen at 13 days, and increased sharply after 20 days. It is of interest that all taxol produced was secreted into the medium, which was very unusual for plant cell cultures. Wickremesinhe and Artheca of the Pennsylvania State University established callus cultures and suspension cultures from T. brevifolia cv Repandens, T. cuspidata, T. media cvs. Hicksii and Densiformis. Though some cell lines grew fast and their doubling times were 9-14 days, the levels of taxol were too low for commercial production. Induction of the hairy roots of Taxus plants has been tried.

DiCosmo and his colleagues of the University of Toronto in collaboration of Nippon Oil Co. Ltd. in Japan described that they detected and identified taxol in the callus of T. cuspidata and its level was 0.02+0.005% in dry weight after 2 months in culture. Suspension cultures of T. cuspidata were also established from the callus cultures and subsequently immobilized onto glass fiber mats. The cells were maintained as immobilized cultures for 6 months. The level of taxol in the immobilized cells was 0.012+0.007% of the extracted dry weight.

Taxol is one of the most suitable and desirable plant products to plant cell culture research because the shortage of its supply and its high-value. Bristol-Myers Squibb is therefore, financially supporting many research groups to establish the process of cell culture production of taxol.

Ginseng

The root of Panax ginsengr, a perennial herb, so-called “ginseng” has been widely used as a tonic and precious medicine since ancient times particularly in oriental countries including Korea and China. It is effective for gastroenteric disorders, diabetes and weak circulation, and has been used as an adjuvant to prevent various disorders, rather than a medicine to cure disorders. Thus, ginseng has been recognized as a miraculous medicine in preserving health and longevity. It has been known that the root contains various saponins and sapogenins. Among them, ginsenoside-Rb has a sedative activity, while Rg has a stimulative activities.

Although there are several species of ginseng, the commercially important species, P. ginseng grows in an area of 30-48^{o} north latitude such as Korea. The cultivation of ginseng in the field requires four to seven years, and it is impossible to plant consecutively for 20 to 50 years but the demand for the plant has increased dramatically in the world, and its price has soared. These are reasons why many researchers have tried to produce ginseng cells through plant tissue cell cultures.

Furuya et al. at Kitasato University have studied P. ginseng callus tissues since the early 1970’s. Meiji Seika Kaisha in Japan investigated the large-scale production of the cells established by Furuya using various types of fermentors. According to their patent published in 1973, crown gall calli, callus tissues and redifferentiated roots of P. ginseng were able to accumulate saponins and sapogenins known from the intact plant. The callus tissues and roots were cultivated on both MS solid and liquid media containing vitamins, sucrose, 2,4-D and suitable natural nutrients such as soybean powder or beef extract for several weeks at 25-28oC. The concentrations of crude saponins in the callus (21.1%), in the crown gall (19.3%) and in the redifferentiated root (27.4%) were much higher than those in the natural root (4.1%). The saponins were found to contain ginsenoside-Rb and -Rg. To obtain high saponin-producing cells, mutagenesis was conducted using nitrosoguanidine and X-ray as mutagens and a variant cell line of the drown gall induced by X-ray irradiation was shown to accumulate 25.5% of saponins.

Staba of the University of Minnesota also obtained cultured cells of P. ginseng containing ginsenosides. Choi of Korea Ginseng & Tobacco Research Institute has investigated in vitro culture of P. ginseng extensively. He indicated that plant growth regulators such as 2,4-D and kinetin in the medium affected the levels of saponins in callus and suspension cultured cells. For example, 3.62% of total saponins was detected in the callus cultivated in MS medium containing 5 mg/L 2,4-D and 1 mg/L

kinetin, while 8.78% was produced in 10 mg/L 2,4-D and 1 mg/L kinetin medium.

After the Meiji Seika abandoned development of the ginseng project, another Japanese company, Nitto Denko Corporation, constructed a 20 KL fermentor to scale-up cultivation of ginseng cells in collaboration with Furuya in the middle of the 1980's. Ushiyama et al. of the company have optimized various environmental conditions using 30 L jar fermentors for the cell line having partly differentiated tissues originally induced by Furuya et al. Glucose in the medium promoted cell growth in the initial stages of the fermentation and sucrose fed during the growth cycle stimulated the productivity of saponins. Although a higher NO_2/NH_3 ratio was favorable to the growth, it decreased the concentration of saponins in the cells. The growth was suppressed by moderate agitation, but the yield of saponins increased. The highest cell mass, 19 g/L on a dry weight basis was obtained using a 2 KL fermentor and the production rate of the cell mass was approximately 700 mg/L/day.

Ushiyama indicated that their cultured cells contained basically the same constituents as those in the intact plant. Acute virulence tests, Ames tests and dietary tests with livestock feed containing 12.5% of dried cultured cells for 6 months did not show any abnormalities in animals.

In 1988, Nitto Denko was approved by the Ministry of Welfare and Health in Japan to market the cultured ginseng cell mass as a food additive. The product has been used as an additive for wines, tonic drinks, soups, herbal liqueurs and others. The company is expecting that the cultured ginseng cells will be approved as a drug by the Ministry within a couple of years.

Rosmarinic Acid

Rosmarinic acid, or a-0-caffeoyl-3,4-dihydroxyphenyllactic acid has been found in the families Laminaceae and Boraginaceae. Rosmarinic acid and some related compounds were reported to have physiological or pharmaceutical activities. Oxidized rosmarinic acid was reported to show antithyrotropic activity and rosmarinic acid itself has been shown to effectively suppress the complement-dependent components of endotoxin shock in rabbits, however these compounds have not yet been used as commercial drugs.

Cultured cells of several plant species such as Coleus blumei, Anchusa officinalis and Lithospermum erythrorhizon were found to accumulate rosmarinic acid. It is of interest that production of the acid is stable, and high levels are produced. Ellis described that the production of rosmarinic acid appeared to be constitutively expressed in C. blumei cells, some of which had been in continuous culture for 10 years with no reduction in metabolite yield.

Dedifferentiated cell cultures of C. blumei and A. officinalis accumulated almost exclusively rosmarinic acid with the levels higher than in the intact plants when both types of cells were cultivated in a Gamborg and Eveleigh's B5 medium. The yield of the acid, before optimization, was about 1.4 g/L for C. blumei and 0.7 g/L for A. officinalis. Both species grew very well and reached up to 16 g-d.w./L within 30 to 40 hours of the culture period.

Zenk et al. reported in 1977 that increasing the sucrose concentration in B5 medium up to 7.5% greatly stimulated both cell growth and rosmarinic acid formation in C. blumei cultures. The yield of the acid was approximately 3.6 g/L and the cell mass was 27 g-d.w./L. The yield shown seems to be one of the highest productivities of secondary metabolites in plant cell cultures.

Culture conditions and correlation between cell growth and production of rosmarinic acid were investigated extensively by De-Eknamkul and Ellis using both cell lines, for example NAA was the most favorable auxin to produce the product for A. officinalis cells, while 2,4-D was the most effective in C. blumei cultures.

Alfermann and his colleagues in Germany have been investigating the biosynthetic pathway of rosmarinic acid and found two new enzymes, i.e., hydroxyphenylpyruvic acid reductase which catalyses the reduction of 4-hydroxyphenyl pyruvic acid to the corresponding lactic acids, and rosmarinic acid synthetase (caffeoyl-CoenzymeA-3,4-dihydroxyphenyllactic acid caffeoyl transferase), the enzyme transferring the caffeoyl moiety from caffeoyl-CoA to 3,4-dihydroxyphenyllactic acid. This is the crucial enzyme in rosmarinic acid biosynthesis forming the ester linkage between the caffeic acid moiety and the 3,4-dihydroxyphenyllactic acid moiety.

To confirm localization of rosmarinic acid accumulated and its biosynthetic enzymes in the cells, the same group prepared protoplasts from C. blumei cultured cells. Rosmarinic acid as well as the enzymes were mainly found in vacuoles. They also purified some of the enzymes.

Recently, Mizukami and Ellis isolated three isoforms of tyrosine aminotransferase (TAT) from Anchusa officinalis cell suspension cultures and indicated two (TAT-1, TAT-4) out of three isoforms were involved in the synthesis of rosmarinic acid. They suggested that TAT-1 controls conversion of tyrosine to 4-hydroxyphenyl pyruvate and TAT-4 acts by participating in the formation of tyrosine and phenylalanine via prephenate.

Determination of the biosynthetic pathway of secondary products will undoubtedly contribute to the further improvement of producing cells using recombinant DNA technology. This technology should be applied more extensively in the future.

Ulbrich et al.of A. Nattermann & Cie. GmbH in Germany investigated large-scale production of rosmarinic acid by a C. blumei cell culture process. In order to increase oxygen supply in the medium and to operate at high cell density, they designed a special module spiral stirrer. It was built of six modules, and a special end module each consisting of one plain metal ring (spiral blade) fixed to the stirrer shaft with two spokes and two rings. The rotation speed of the module spiral stirrer ranged between 50-100 rpm without significant cell damage. Standard rotation speed was set at 100 rpm. The inoculum of C. blumei cells was cultivated in a fed-batch process and then about 30-50% culture broth was transferred from the seed fermentor to the production fermentor with sucrose solution (50 g/L) as the production medium.

Using this procedure the yield of rosmarinic acid increased to 5.5 g/L or 910 mg/L/day, representing 21% of the dry weight. According to Ulbrich's experiments, the process needs only 14 days to produce in total 200 g of 97% purity of rosmarinic acid in two parallel production batches from one inoculum fermentor (32 L working volume). It is not necessary to separate the cell mass from the growth medium, and they would recycle it as a part (30% v/v) of the simple and cheap production medium (sucrose 50 g/L). It is hoped that some commercial utility for rosmarinic acid will be found in the future.

Arbutin

Arbutin is widely distributed in various plants of the Ericaceae such as Arctostaphylos uva-ursi and Vaccinium vitisidaea. The level of arbutin in the bark of Pyrus communis reaches up to 28%. Arctostaphylos uva-ursi has been used as a urethal disinfectant and its major active principle, arbutin, was shown to suppress the synthesis of melanin in human skin. A Japanese cosmetic company, Shiseido, has developed arbutin as an additive for the company's product lines because of its preventive activity toward pigmentation of skin.

Although arbutin is commercially available by a chemical process, researchers of Shiseido have investigated an alternative process using plant cell cultures including biotransformation. Tabata et al. showed that cultured cells of Datura innoxia had a remarkably high capability for glucosylation of hydroquinone to form arbutin, and hydroquinone was totally converted to arbutin within 10 hours after administration. The glucosylation is catalyzed by an enzyme, uridine diphosphate glucose (UDPG)-hydroquinone glucosyltransferase. Yokoyama et al. selected C. roseus cells as a producer of the enzyme since arbutin was formed efficiently when hydroquinone was added into the suspension culture.

They have optimized various components in Linsmaier-Skoog's basal medium for production of arbutin and found that higher levels of sugars such as sucrose in the medium, up to 6%, gave higher yields of arbutin. Concerning this sugar effect, Yokoyama suggested that sucrose was a scavenger, and therefore it protected cultured cells from the damaging activity of hydroxy radicals of the substrate. This was also supported by their finding that some antioxidants such as ascorbic acid, gallic acid, cysteine, tannin and phytic acid increased the level of arbutin.

One of the high producing cell lines, C. roseus strain B was cultivated in a 5 L jar fermentor equipped with modified paddle-type impellers and spargers of porous sintered metal which prevents to some extent the adhesion of cells to the inner surface of the fermentor. Glucose was used as a carbon source instead of sucrose for economic reasons for mass production of arbutin. In order to increase the cell density in the medium, 10 times concentration of the medium components was fed to the medium during the fermentation. It was prerequisite to keep the level of hydroquinone in the medium as low as possible to avoid damage of the cells by the substrate. Therefore, hydroquinone was fed at 6 mM in the beginning of the fermentation and after its concentration had decreased to around 0 mM in the medium, they started to feed hydroquinone continuously at 1.4 mmol per hour. Under these conditions, 9.2 g/L of arbutin which corresponded to 45% of dry cell weight was obtained in 3 to 4 days after administration of hydroquinone. Similar yields was also obtained using larger scale fermentors.

Since the production continued until cell death, arbutin was accumulated extracellularly and its concentration reached approximately 1% in the culture filtrate at the end of the production phase. Therefore, arbutin was easily extracted from the filtrate. The total cultivation period was approximately 18 days including 2 weeks for high density cell culture and 3 or 4 days for the biotransformation process.

According to Yokoyama, the chemical synthesis of the compounds requires at least 3 step reactions and the production cost of arbutin by a plant cell culture process is comparable to the chemical process.

As described previously, biotransformation is one of the most feasible processes in terms of industrial application of plant tissue and cell cultures. It is advantageous that the yield of arbutin is high and the cost of hydroquinone is inexpensive enough as a substrate, accordingly the author believes that the process will be employed for commercial manufacturing arbutin in the near future.

Conclusion

The detailed account of biochemicals given in the Lesson has proved that higher plants are the source of a large number of pharmaceutically important biochemicals. About 25% of the prescribed medicines are solely derived from plants. Many of these pharmaceutical compounds have complex structures which makes their chemical synthesis economically unattractive. Some compounds like diterpene alkaloid taxol (1g taxol from 7 kg dried bark of Taxus), trichosanthin and karasurin (both proteins) have extremely valuable properties. Recently, a taxol derivative (Taxotere) has been taken to clinical trials. Shikonin is also produced on a commercial scale from cell cultures. Hence, we can conclude that biochemicals obtained from plant cell cultures are comparable to those derived from intact plants in their chemical, biochemical and biological properties.

Questions

1. Briefly describe the various alkaloids obtained through plant cell cultures.
2. Give a brief account of the anti tumour compounds obtained from plants.
3. Write a short note on Valepotriates.

Note